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13. ABSTRACT (Maximum 200 Words)

New contrast-specific imaging modalities such as harmonic imaging (HI) may improve the accuracy of breast ultrasound. Unfortunately, HI suffers from reduced blood-to-tissue contrast resulting from second harmonic generation and accumulation in tissue. As an alternative we propose using subharmonic imaging (SHI) by transmitting at the double the resonance frequency ($2f_o$) and receiving at the subharmonic (f_o). SHI has the potential to detect slow, small volume blood flow associated with tumor neovascularity, making early detection and identification of tumors very likely. Hence, the current project proposes to increase the ability of breast ultrasound to differentiate between benign and malignant lesions by combining injection of an ultrasound contrast agent with SHI.

To date, in vivo experiments comparing SHI in canines to perfusion have been completed showing good correlation ($r = 0.57$; $p < 0.0001$) with contrast uptake slopes. Otherwise the entire year has been devoted to obtaining the necessary approvals from collaborators as well as regulatory bodies (internal as well as external) in order to commence the human studies. Currently final approvals are expected to be in place by early August, 2004.

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3. TABLE OF CONTENTS

4. INTRODUCTION

The goal of any breast imaging modality is to improve the early detection of tumors and to improve the differentiation between benign and malignant lesions. While x-ray mammography is efficacious in diagnosing a high percentage of breast masses, it also produces a high rate of false positives [1]. The percentage of breast biopsies that are actually malignant vary between 10 % and 35 %. Thus, a technique that reliably differentiates between malignant and benign masses would improve the diagnosis of breast cancer and should, therefore, reduce the number of negative biopsies as well as the trauma of the patients. This proposal will attempt to establish such a technique through the novel and innovative use of subharmonic ultrasound contrast imaging.

Ultrasound imaging is currently an auxiliary modality in breast imaging. It is mainly used to differentiate between cystic and solid lesions [2]. Investigations into the possibility of breast cancer diagnosis based on Doppler ultrasound flow detection have produced mixed results, due to overlap between flow measurements in benign and malignant tumors [3-4]. One problem may be the lack of sensitivity in flow detection in small tumor vessels using ultrasound. This hypothesis is supported by reports in the pathology literature describing angiogenic vascular morphology as an independent predictor of metastatic disease [5].

Ultrasound contrast agents produce increases of 15 to 25 dB in the echo intensities of blood flow signals; especially when combined with new contrast-specific imaging modalities such as harmonic imaging [6-7]. However, harmonic imaging has been found to suffer from reduced blood-to-tissue contrast resulting from second harmonic generation and accumulation in tissue. As an alternative we propose using subharmonic imaging (SHI) by transmitting at the double the resonance frequency ($2f_o$) and receiving at the subharmonic (f_o). SHI has the potential to detect slow, small volume blood flow associated with tumor neovascularity, making early detection and identification of tumors very likely. SHI should have much better lateral resolution due to the higher transmitting frequency and should allow tumor perfusion, a measure of angiogenesis, to be estimated via time-dependent subharmonic fractional blood volume estimates. Hence, the current project proposes to increase the ability of breast ultrasound to differentiate between benign and malignant lesions by using SHI.

Quantifiable parameters of tumor angiogenesis will be estimated from the subharmonic signal intensities. A pulse-echo system will be built to perform SHI and tested in vitro as well as in vivo (in animals). The ability of SHI to depict normal vascularity as well as tumor angiogenesis will also be assessed in rabbits. Currently, the NIH and DOD have funded a study at Thomas Jefferson University into the efficacy of ultrasound contrast in the diagnosis of breast disease. We propose to expand on that project by adding SHI in the third year of this proposal. Not only is the potential of SHI in itself innovative, but because of the NIH/DOD funded study it will be possible to compare a number of new and unique approaches to breast cancer diagnosis i.e., SHI, 2D power Doppler with and without contrast as well as harmonic imaging directly to x-ray mammography. Furthermore, this project is extremely cost-effective because the existing grants coves a majority of the personnel costs as well as all major equipment purchases. The amalgamation of the NIH/DOD project with the current proposal also allows for basic research

into the correlation between SHI flow signals and pathologically detected lesion vascularity. This will enable a deeper understanding of the relationship between tumor neovascularity and ultrasound flow measurements

Consequently, this project proposes the development of a novel contrast specific imaging mode called SHI and the derivation of quantitative tumor angiogenesis estimates from SHI data. The fundamental hypothesis is that the neovasculature of malignant lesions can be visualized and quantified with SHI, thus, improving the diagnosis of breast cancer.

5. BODY

The central hypothesis of this project is that the differentiation between benign and malignant breast lesions can be improved by detection and estimation of tumor neovascularity using contrast enhanced SHI. To investigate this hypothesis SHI will be investigated in vitro and then in vivo in rabbits with VX-2 tumors. Finally, approximately 50 women with breast lesions will be recruited in year three and imaged using contrast enhanced SHI. The specific tasks of the project (as presented in the original Statement of Work) can be found in Appendix I.

First an outline of the methods applied will be given followed by a presentation of the results to date. Finally, the conclusions and future directions of the research will be discussed.

5.1 Methods

In vivo experiments

Four laboratory bred mongrel dogs (mean weight 21 kg) were used in this project. The dogs were premedicated with intramuscular administration of a mixture of 0.04 mg/kg atropine sulfate (Anthony Products, Arcadia, CA), and 0.75 mg/kg acepromazine (Promace; Aveco, Fort Dodge, IA). The dogs were placed on a warming blanket to maintain body temperature within normal range. A facemask with Isoflurane 4-5 % (Iso-thesia; Abbott Labs, N.Chicago, IL) was used for induction of anesthesia, which was maintained with 0.5 to 2 % of Isoflurane during the entire procedure. The animal studies were performed under supervision of a veterinarian and fully conformed to the National Institutes of Health guidelines for use of laboratory animals. All protocols were approved by the University's Animal Use and Care Committee.

Bilateral renal grayscale SHI was performed using a modified Logiq 9 scanner (GE Healthcare, Milwaukee WI) with a 7L probe. The transmission frequency was 4.4 MHz and the receive frequency was 2.2 MHz. The dogs received intravenous contrast injections of the contrast agent Optison (GE Healthcare, Princeton, NJ) at a dosage of 0.1 ml/kg. Following three contrast injections, a microvascular staining technique based on stable (non-radioactive), isotope labeled microspheres (BioPhysics Assay Laboratory Inc, Worcester, MA) was employed to quantify the degree of perfusion. Next, low perfusion states were induced by ligating surgically exposed segmental renal arteries. The contrast injections as well as SHI were repeated followed by administration of more isotope labeled microspheres (different metals for the right and left side). At the completion of the experiments, the dogs were sacrificed using an intravenous injection of Beuthanasia (0.25 mg/kg).

After euthanasia, the kidneys were harvested, cut into eight sections (upper pole anterior and posterior, mid-upper pole anterior, ..., lower pole posterior) and weighed. The tissues were exposed to gamma radiation (at BioPal's facilities) and the spheres temporarily became radioactive. The activity was determined using spectroscopy. The ratio of the number of spheres in the tissue sample to that in a timed reference blood sample provides an absolute measure of perfusion (Q_p; in ml/min g):

$$Q_P = \frac{\sum spheres\ in tissue \Big/ \sum spheres\ in reference}{weight\ of\ tissue} \quad (1)$$

Digital clips of each SHI injection were transferred to a PC for off-line analysis. SHI time intensity curves were acquired in each of the eight kidney sections using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). SHI fractional blood volumes (FBVs) were calculated as the SHI signal intensity normalized by the intensity of 100 % blood (i.e., a normal blood vessel) and smoothed with a fifth-order moving average (MA) filter using Matlab (The MathWorks Inc, Natick, MA). Perfusion was estimated from the initial slope of the FBV uptake (rFBV) as:

$$\text{rFBV} = d\text{FBV}/dt \quad (2)$$

Perfusion estimates were avareaged over three injections to obtain the final result. SHI perfusion data was compared to the gold standard microvascular staining technique using linear regression analysis with p-values less than 0.05 considered significant. Finally, the perfusion data was split into different locations (anterior and posterior) and different perfusion states (high and low i.e., before and after ligation) as well as the four possible combinations of location and perfusion state. Then the statistical analysis was repeated.

5.2 Results and Discussion

Canine in vivo experiments

In vivo SHI with Optison clearly demonstrated perfusion in the kidney as shown in Figure 1. Notice the excellent suppression of tissue echoes. This suppression of tissue signals is even more marked on the baseline image (i.e., prior to administration of Optison), which is completely black (image not included for the sake of brevity). The transmission frequency was 4.4 MHz and the receive frequency was 2.2 MHz.

A total of 270 SHI time intensity curves were acquired, which reduced to 94 perfusion estimates after averaging. There were 18 curves, which were eliminated due to technical failures (mainly shadowing from the anterior portion of the kidneys and very low perfusion states). An example of an original time-intensity curve and the one obtained after smoothing with the fifth-order MA filter is presented in Figure 2. Overall the SHI perfusion estimates correlated significantly with microsphere results ($r = 0.57$; $p < 0.0001$). The complete perfusion data set as well as the best linear fit ($Q_p = 0.47$ rFBV + 1.62) is depicted in Figure 3. The corresponding root-mean-square-error was 2.48 %.

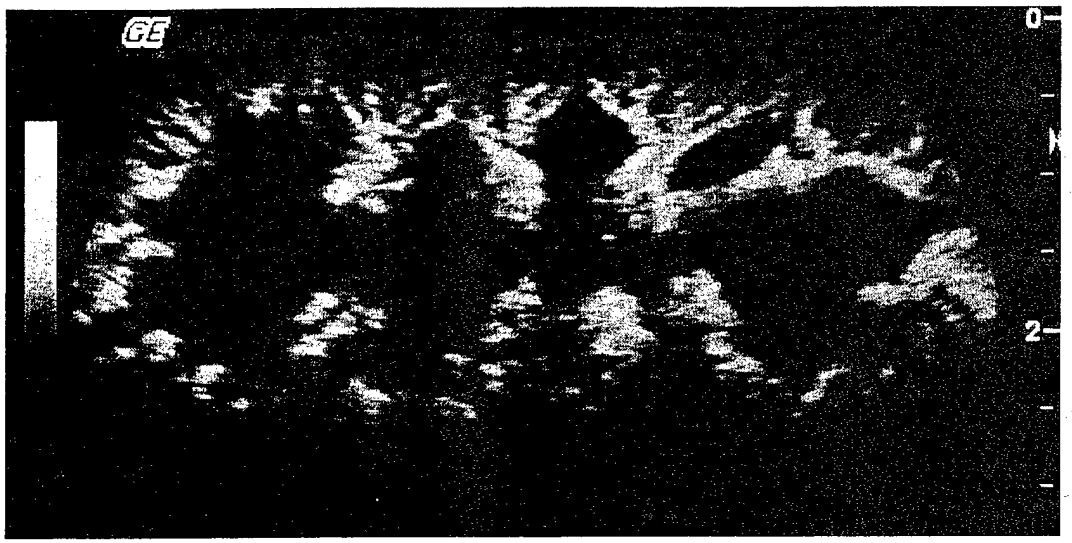

Figure 1. *In vivo SHI post injection of 0.1 ml/kg of Optison showing perfusion in the left kidney obtained with the modified Logiq 9 scanner. The pre injection image is completely black (no subharmonic flow signals are detectable before the microbubbles arrive) and has, therefore, been omitted.*

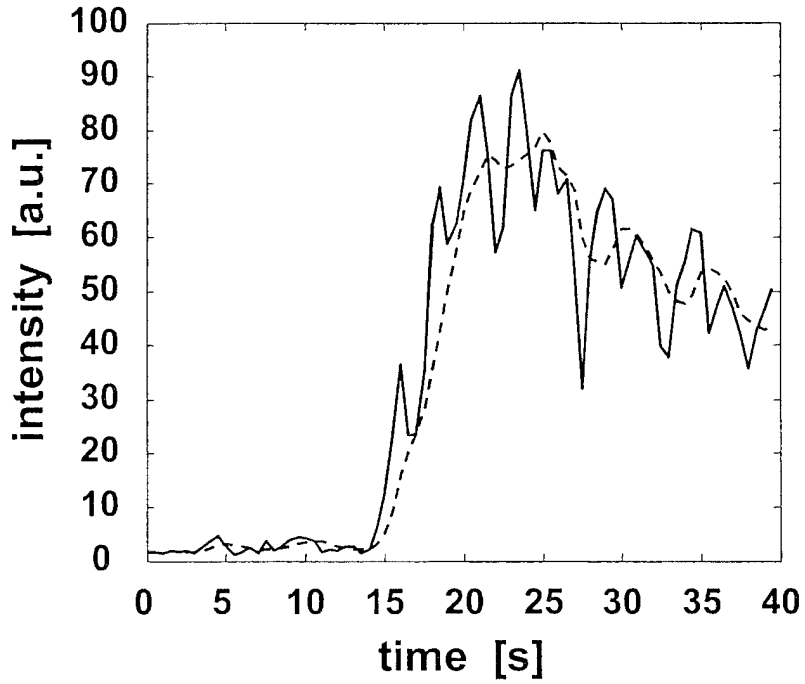

Figure 2. *SHI time-intensity curves before (i.e., original data; solid line) and after smoothing with the fifth-order MA filter (dashed line). The arrival of contrast bubbles approximately 14 s after injection can clearly be seen*

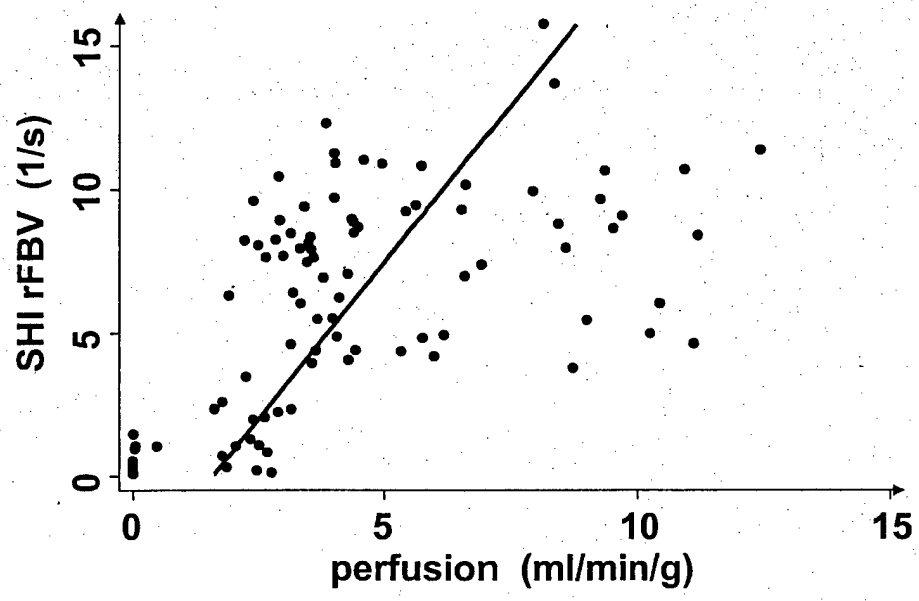

Figure 3. *The microsphere perfusion (in ml/min g) versus all SHI perfusion estimates as well as the best linear fit (solid line).*

When the SHI perfusion estimates were split into subsets and the linear regression analysis repeated, significant correlations with the gold standard (i.e., the microsphere results) were found ($0.47 < r < 0.74$; $p < 0.022$) as shown in Table 1. The best SHI perfusion estimates occurred for high perfusion states in the anterior of the kidneys ($r = 0.73$; $p = 0.0001$); albeit based on a limited data set (N=22). In other words, when the acoustic shadowing was minimal and the concentration of contrast microbubbles was the highest, SHI perfusion estimates were most accurate. Notice, that even if Bonferroni correction was applied [8], only high perfusion in the posterior of the kidneys would not produce a significant correlation. This work has been accepted for publication [9].

Table 1. Sub-analyses of the SHI perfusion data.

	r value	p value
Anterior	0.56	< 0.0001
Posterior	0.57	< 0.0001
High perfusion	0.61	< 0.0001
Low perfusion	0.64	< 0.0001
Anterior / high	0.73	0.0001
Anterior / low	0.57	0.0037
Posterior / high	0.48	0.0214
Posterior / low	0.71	0.0001

There are some limitations to the current study apart from the problems associated with acoustic shadowing and low perfusion states (as mentioned previously). Motion artifacts induced by the dogs breathing were not compensated for when the time-intensity curves were acquired (although the ROI's were chosen large enough to reduce such effects). More importantly, the SHI data were obtained from a single imaging plane and this will induce some additional variability when compared to the microvascular staining technique, which is inherently three-dimensional in nature. To somewhat compensate for this problem, care was taken to both image the largest diameter of the kidneys and to cut the harvested kidneys in that largest diameter (however, some variability is inevitable).

Human in vivo experiments

In order to obtain IRB approval for the human component of this project the acoustic power levels of the SHI software on the selected probe (the 7L) was measured. This effort was carried out at GE Medical Systems in Milwaukee during the week of August 11th, 2003. By then all technical and scientific aspects required to enable the initiation of the human clinical trial were completed (as planned and reported in the Annual report of 2003). All subsequent efforts have been focused on the administrative work required to initiate the trial.

Research agreements between Thomas Jefferson University and GE Medical Systems and Amersham Health, which were required to conduct the human clinical trial at Thomas Jefferson University, took three months to finalize. Following receipt of the fully executed agreements in mid-November, the submission for the Human Subjects Research Review Board (HSRRB) was prepared. The Thomas Jefferson University Institutional Review Board approved protocol, consent form, and case report forms were submitted to the U.S. Army Medical Research and Material Command on December 22, 2003, but the protocol was not reviewed by HSRRB until March 10th, 2004. Comments from the HSRRB meeting and suggested minor changes were emailed to the Principal Investigator on April 9th. The revisions received final approval by the University's Institutional Review Board in early June, 2004 and was submitted to the HSRRB by June 16th, 2004. We are currently awaiting final approval by the HSRRB and, hence, permission to commence the human clinical trial. This final approval is expected by late July or early August, 2004. These efforts represent the commencement of task 3a.

Consequently, eleven months that were planned for conducting the clinical trial has instead been devoted to obtaining all of the necessary administrative approvals, leaving no time to conduct the actual clinical trial. For that reason, we requested an additional one year no cost extension to recruit patients for participation in the clinical trial and, thus, for completing the goals of the project.

6. KEY RESEARCH ACCOMPLISHMENTS

- SHI perfusion studies were conducted in canines.
- In vivo, 270 SHI time intensity curves were acquired.
- From this data set, 94 SHI perfusion estimates were obtained.

- The best SHI perfusion estimates were found for high perfusion states in the anterior of the kidneys ($r = 0.73$; $p = 0.0001$); albeit based on a limited data set.
- SHI perfusion estimates correlated significantly with microsphere results ($r=0.57$; $p<0.0001$).
- After 11 months the approval process for the human subject study is **almost** complete.

7. REPORTABLE OUTCOMES

G Bhagavatheeshwaran, WT Shi, F Forsberg, PM Shankar. Subharmonic generation from contrast agents in simulated neovessels. *Ultrasound Med Biol*, vol. 30, no. 2, pp. 199 – 203, 2004.

F Forsberg, JB Liu, WT Shi, R Ro, KM James, X Deng, AL Hall. Perfusion estimation using subharmonic contrast microbubble signals. Accepted for publication in *Proc IEEE US Symp*, 2004.

September 25, 2003	Breakthrough Seminar 2003, GE Yokogawa Medical Systems, Osaka, Japan. • New Methods and Applications for Ultrasound Contrast Imaging.
September 27, 2003	15th Doppler Ultrasound Meeting, Japanese Ultrasound Society, Tokyo, Japan. • Recent Developments in Contrast Enhanced Ultrasound Imaging – an American Perspective.
October 24, 2003	Biomedical Ultrasound Faculty Group Seminar, Drexel University, Philadelphia, PA, USA. • In Vivo Subharmonic Imaging and Pressure Estimation.
May 11 - 14, 2004	The Leading Edge in Diagnostic Ultrasound, Atlantic City, NJ, USA. • In Vivo Subharmonic Imaging and Perfusion Estimation.

8. CONCLUSIONS

The in vivo animal experiments have been completed (i.e., task 2). The SHI perfusion estimates were in reasonable agreement with a microvascular staining technique ($r = 0.57$; $p < 0.0001$; Fig. 3). Moreover, the best SHI perfusion estimates were found for high perfusion states in the anterior of the kidneys ($r = 0.73$; $p = 0.0001$; Table 1); albeit based on a limited data set.

The acoustic power testing of the SHI software on the 7L probe was completed at GE's testing facility in August 2003. All subsequent efforts were focused on the administrative work required

to initiate the clinical trial of SHI in women with breast lesions. We are currently awaiting final approval by the HSRRB and, hence, permission to commence the human clinical trial. This final approval is expected by late July or early August, 2004.

In summary, tasks 2a, 2b and 2c have been completed while task 3a is ongoing, but due to the delay caused by the approval process for the human subject study the project is approximately 12 months behind schedule. Another one year no cost extension has consequently been requested (and granted).

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Appendix I

The Statement of Work from the original proposal:

Objectives 1 - 2
Task 1: Software development and *in vitro* experiments (months 1 - 24)

a. Develop software for SHI and for FBV estimates to be produced from SHI data (months 1 - 24).
b. Design and implement pulse echo SHI setup (months 1 - 6).
c. Perform *in vitro* flow phantom experiments comparing SHI and FBV estimates to absolute perfusion and flow rates (months 6 - 12).

Objectives 2 - 3
Task 2: Animal experiments and data collection (months 13 - 24)

a. Perform *in vivo* experiments in 12 normal rabbits comparing FBV estimates to absolute flow rates and perfusion obtained with colored microspheres (months 13 - 20).
b. Perform *in vivo* experiments in 6 rabbits with renal VX-2 tumors implanted comparing FBV estimates to absolute tumor perfusion obtained with colored microspheres (months 20 - 24).
c. Evaluate the performance of SHI in the detection of rabbit VX-2 tumors compared to conventional ultrasound imaging, with and without contrast administration, as well as to harmonic imaging (months 13 - 24).

Objectives 4 - 5
Task 3: Human data collection and analysis (months 25 - 36)

a. Recruit 50 - 75 patients, which is about two-thirds of the anticipated number of patients being enrolled in the existing NIH/DOD supported contrast study (months 25 - 36).
b. Perform SHI contrast studies as part of the already funded NIH/DOD project. This involves an extra injection of contrast (within the permitted total dose) and will add no more than 20 minutes to the total duration of the contrast study (months 25 - 36).
c. Research coordinator to collect clinical information, pathology results, etc. (months 25 - 36).
d. Incorporate SHI findings into the existing database developed for the NIH/DOD supported study (months 25 - 36).
e. Perform ROC analysis in collaboration with the statistician (months 30 - 36).
f. Perform remaining statistical analysis in collaboration with the statistician (months 30 - 36).

Appendix II

Reprint of the peer reviewed manuscript published in Ultrasound in Medicine and Biology:

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● *Original Contribution*

SUBHARMONIC SIGNAL GENERATION FROM CONTRAST AGENTS IN SIMULATED NEOVESSELS

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Abstract—Detection and measurement of blood flow in neovessels around a tumor can yield prognostic information about the tumor. Early detection and classification may help differentiate benign and malignant tumors; thus, improving patient management. This can be accomplished by injecting ultrasonic contrast agents and measuring the backscattered signals from them. Use of the subharmonic backscattered signals from these agents may be better than fundamental or second harmonic components because of the negligible subharmonics generated by the surrounding tissue. Preliminary results on the detection and measurement of subharmonic signal components up to 12 dB (at increasing pressures) from very small tubes (200 to 300 μm diameter) are reported, demonstrating the possibility and potential application of subharmonic imaging in detecting tumor angiogenesis. (E-mail: pshankar@coe.drexel.edu) © 2004 World Federation for Ultrasound in Medicine & Biology.

Key Words: **Tumor angiogenesis, Contrast agents, Subharmonic signals.**

INTRODUCTION

Angiogenesis, the formation of new blood vessels, in and around a tumor is thought to be a direct result of increased levels of vascular growth factors released by the tumor. This formation of new vessels associated with malignant tumors is a precursor to the proliferation of the tumor cells and starts when the tumor is about 1 to 2 mm in diameter (Folkman and Klagsbrun 1987; Li 2000). These microvessels carry nutrients required for the proliferation of the tumor cells, and also provide the means by which the tumor cells can relocate to other distant parts of the body (Folkman et al. 1963; Folkman and Klagsbrun 1987). Hence, angiogenesis is an important characteristic of malignant tumors and its detection is likely to be vital in cancer diagnosis and prognosis.

The role of tumor neovascularity and its proliferation has been studied since 1963 (Folkman et al. 1963). Some of these neovessels grow to radii of 150 to 200 μm (Folkman and Klagsbrun 1987). Although such vessels are common with malignant tumors, their numbers are small or negligible in benign tumors. The detection of such small vessels is difficult because of the insufficient resolution of even state-of-the-art imaging equipment. Many techniques are being used to image neovasculature, including contrast studies with ultrasound (US), computerized tomography (CT) and magnetic resonance imaging (MRI) (Li 2000). Of these techniques, US imaging may offer specific advantages because of its ability to image different levels of vascularity and to pick up very low flow velocities (2 mm/s) (Ferrara et al. 2000). Use of contrast agents will further enhance this capacity.

US contrast agents are gas-filled microbubbles in 1 to 10 μm diameter (Goldberg et al. 2001). The surfaces of these microbubbles are stabilized with surfactants or coated with an elastic shell. The small size of these microbubbles allows them to traverse the capillaries and lungs. The gas in the bubble has relatively low acoustic impedance and a large impedance mismatch is, thus, created with the surrounding blood and tissue. This leads to strong scattering from the contrast agent (in excess of 25 dB over noncontrast pulse echo systems), increasing the sensitivity of the US scanner.

Apart from reflections, microbubbles also exhibit nonlinear vibrations under insonation. When insonified

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with a signal at its resonance frequency (f_0), contrast agents generate signals at f_0, its harmonics $2f_0$, $3f_0$, $4f_0$, etc., and subharmonics at $f_0/2$, $f_0/3$, etc. (Forsberg et al. 1996; Shankar et al. 1998). Diagnostic US propagating through the tissues also generates signals at harmonic frequencies of $2f_0$, $3f_0$, $4f_0$, etc. The amplitude of these harmonic signals from tissue is much lower than that from microbubbles due to the very weak nonlinear properties of the tissue compared to that of the bubbles (Eller and Flynn 1969; Prosperetti 1971; Leighton 1994). Subharmonic backscatter is generated in a medium only when a certain threshold acoustic pressure is exceeded (Prosperetti 1971). In tissue, overcoming this threshold requires high acoustic pressures and, hence, subharmonics in tissue are generally nonexistent under normal acoustic pressures (Shankar et al. 1999; Forsberg et al. 2000).

The threshold for subharmonic generation in microbubbles is relatively low and increases with damping of the coated microbubble. This threshold is found to be a minimum when using an insonation frequency twice the resonant frequency of the microbubble (Eller and Flynn 1969; Prosperetti 1971; Shankar et al. 1999). Thus, the subharmonic signals will have a higher signal-to-background noise ratio than the second harmonics (Shankar at al. 1998; Shi et al. 1999; Shi and Forsberg 2000).

This paper explores the generation and detection of subharmonic signals from a contrast agent flowing through tubes with internal diameters of less than 300 μm, simulating tumor neovascularity. Conditions such as the threshold acoustic pressure for generation of subharmonic backscatter amplitude were studied so that the results could be applied to future *in vivo* tests on subharmonic imaging of angiogenesis.

MATERIALS AND METHODS

In the experiments, individual vessels were simulated (Hindle and Perkins 1994; Veltmann et al 2002) with two types of tubes. The tubes used to set up a flow system were polyester shrink tubes (manufactured by Advanced Polymers Inc., Salem, NH, USA) of two different diameters. The smaller tube had an internal diameter of 300 $\pm$ 25 μm, and a wall thickness of about 6 μm and the larger had internal diameter of 1000 $\pm$ 25 μm and a similar wall thickness.

A dual transducer setup was used for transmitting and receiving pulse echo US signals. The contrast agent used for the study was Optison™ (manufactured by Mallinckrodt Inc., St. Louis, MO and copromoted by Amersham Health, Princeton, NJ). The frequency of insonation was 4 MHz, because the resonance frequency of Optison™ is around 2 MHz (Shi et al. 1999; Forsberg et al. 2000). This is in keeping with the concept of a minimum threshold for subharmonic generation when the insonation frequency is twice the resonance frequency.

A pulse/function generator (8111A; Hewlett-Packard, Palo Alto, CA) was used to generate 32 cycle busts with a pulse repetition rate (PRF) ranging between 20 and 100 Hz. A radiofrequency (RF) power amplifier (A150; ENI Technology Inc., Rochester, NY) amplified this signal by 55 dB to generate pressure levels from 0.3 to 1.5 MPa. The transmitting narrow band width transducer had a center frequency of 5 MHz and focal length of 2.54 cm (13-0508S; Harisonic/Staveley Industries plc, Croyden, UK). The backscattered signals were picked up using a wideband transducer (2.54 cm focal length) with center frequency of 2.25 MHz (13-0208R; Harisonic/Staveley). The acoustic pressures were measured using a calibrated 0.5-mm miniature needle hydrophone (Precision Acoustics, Dorchester, UK).

The US was focused onto the thin tube (300 μm) embedded in a phantom to ensure that the tube did not move during the experiment. The homogenous phantom used for this experiment was made from acrylamide gel. The choice of this material was based on previous work with ultrasonic phantoms (Narayanan et al. 1994). To make the phantom, a mold was first made with the tube running through it. A solution of 150 mL of distilled deionized water, 7.5 g of acrylamide and 0.36 g of bis-acrylamide was prepared separately. To this solution, 36 mg of ammonium persulphate was dissolved carefully. This solution was then poured carefully into the mold to avoid formation of any air bubbles on the tube. A micropipette was used to add 66 μL of N,N,N′,-N′-tetramethylethylenediamine (TMED) to the solution, and it was allowed to polymerize, forming the phantom. After it was fully polymerized, the phantom with the tube embedded in it was carefully taken out of the mold and placed in the water tank for the experiment.

The receive transducer was placed at an angle of 40° to the transmit transducer. First, care was taken to ensure that the focal region of the two transducers overlapped. The next step was to ensure that the tube lay within this overlap region. This was verified by filling the tube with air and adjusting the spatial alignment of the phantom until the maximum reflection from the tube was obtained. For reproducibility of the results, a 20 V pk-pk input burst was applied (based on the needle hydrophone used, this corresponded to an acoustic pressure of 0.2525 MPa), and the position of the air-filled tube was adjusted until the reflected signal amplitude was around 20 mV for the larger diameter tube. For the smaller tube, a reflected signal of 8 mV was the best that could be achieved with any reliability. Every effort was made to ensure that the temperature in the water tank was constant (~25°C).

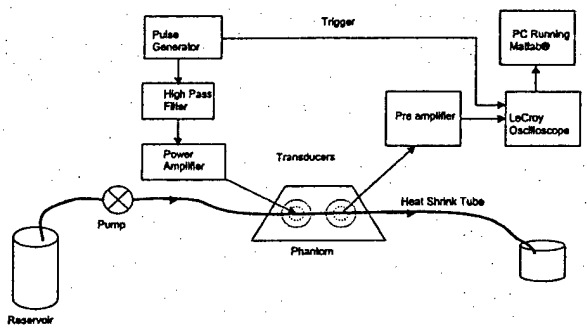

Fig. 1. Setup used for the experiment.

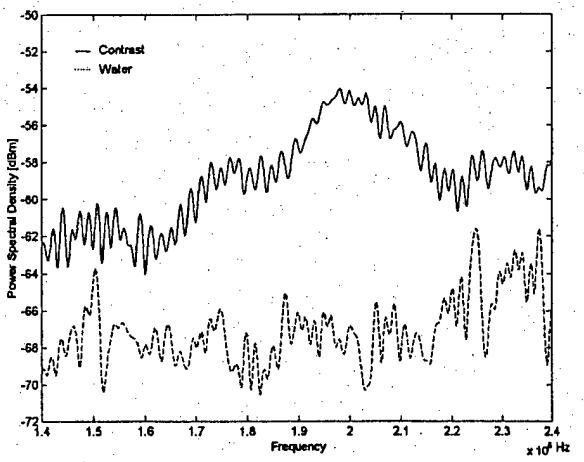

Fig. 2. Power spectral density averaged over of 16 waveforms. The waveforms correspond to backscatter from contrast agent and distilled water, flowing through a tube with internal diameter of 300 μm. The insonation power was 1.5 MPa with a PRF of 50 Hz and insonation frequency of 4 MHz.

The signal received by the transducer was captured using a digital oscilloscope (9450A; LeCroy, Chestnut Ridge, NY) through a preamplifier with 20-dB gain. An oscilloscope sampling frequency of 40 MHz was used. Data were then transferred to a PC, and software written in Matlab® (The Mathworks, Natick, MA) was used to calculate the power spectral density of the signal.

A pump driver (Masterflex; Cole Parmer Instrument Co., Vernon Hills, IL) with pulsatile flow velocity and adjustable rates of flow was used to control the flow in the tube. A 20-gauge needle was used to inject the contrast into the larger polyester shrink (1000 μm) tube. Similarly, a 32-gauge needle was used for the thinner tube (300 μm). The setup is shown in Fig. 1.

Optison™ was used at a dilution of 0.67 mL/L of water. This concentration is equivalent to the dilution expected after an injection of 4 mL of contrast agent in an adult male. Distilled deionized water was used to dilute the contrast agent, as well as for the control for the experiment. The average velocity of flow of water in the tube was calculated from measuring the volume of water that was collected in a beaker over time and dividing by the area of cross-section of the tube.

Maximum amplitude of the power spectral density (PSD) computed between 1.9 MHz and 2.1 MHz was measured as the subharmonic amplitude when an insonation frequency of 4 MHz was used with Optison™. A total of 16 waveforms were acquired for each insonation level, and five different insonation levels between 0.3 and 1.5 MPa were used. The subharmonic component of each waveform acquired was used to calculate the average subharmonic generated at that pressure level.

In the human body, angiogenesis gives rise to many tortuous vessels. To test results in a more realistic situation and to simulate conditions similar to the flow of blood in these abnormal tissues, we used a high-efficiency single-use dialyzer (F7NR; Fresenius, Bad Homburg, Germany). This cartridge consists of a few thousand very thin tubes encased in a longer plastic tube. The internal diameter of each of these tubes is 200 μm and they have a wall thickness of 6 μm. An acoustic window was cut into the outer tube to prevent any attenuation. Such perfusion phantoms have been used by other researchers as well (Hindle and Perkins 1994; Veltmann et al. 2002).

The experiments were repeated with a dialysis cartridge replacing the polyester tube. The transducers were focused on the hollow tubes directly through the acoustic window of the dialyzer. The dialysate compartment and the entire setup were immersed in water. Care was taken to remove all air bubbles trapped in between the tubes. However, complete removal could not be guaranteed.

A high-pass filter (Krohn-Hite, with a cut-off at 2.3 MHz) was used at the input side, before the power amplifier to reduce any 2-MHz side bands that might have been present. Its effectiveness was confirmed by the absence of any subharmonic component in the backscattered echo from water with varying acoustic pressures. The flow velocity through the hollow tubes of the dialyzer was estimated to about 0.2 cm/s. Backscatter signals from water and contrast agent were acquired. The subharmonic components were calculated using Matlab®.

RESULTS AND DISCUSSION

Figure 2 shows a comparison of the average subharmonic backscatter from the contrast agent and distilled water in a single tube with internal diameter of 300 μm at an acoustic power of 1.5 MPa. Subharmonic signals from the contrast medium could be observed over a

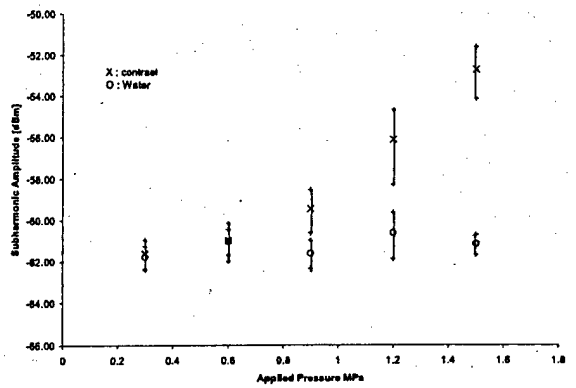

Fig. 3. The variation in the average subharmonic backscatter from the agent and water as a function of the applied pressure. The tube diameter is 300 μm. The vertical bars indicate $\pm$ 1 SD.

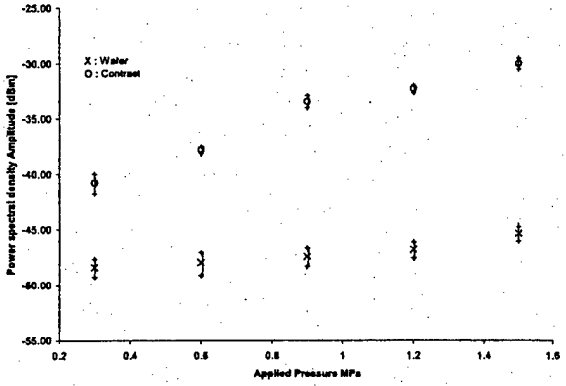

Fig. 5. The variation in the average subharmonic backscatter from the agent and water as a function of the applied pressure from a dialyzer. The internal diameter of the dialyzer tube is 200 μm. The vertical bars indicate $\pm$ 1 SD.

range of frequencies around 2 MHz, with the peak signal close to 2 MHz. The amplitude of the subharmonic backscatter was about 10 dB higher than the signal from distilled water at this pressure level.

Figures 3 and 4 show the average subharmonic backscatter detected for various acoustic pressure amplitudes from contrast flowing through the tube with an internal diameter of 300 μm and 1000 μm, respectively. The figures also provide the average backscatter measured from distilled water flowing through the tube for comparison. A higher subharmonic backscatter could be observed in the larger tube, even at lower acoustic powers. This could be due to the larger volume of contrast medium flowing through the focal region of the transducers in case of the larger tube.

The tube thickness of 300 μm is at the higher end of the diameter of blood vessels formed around tumors.

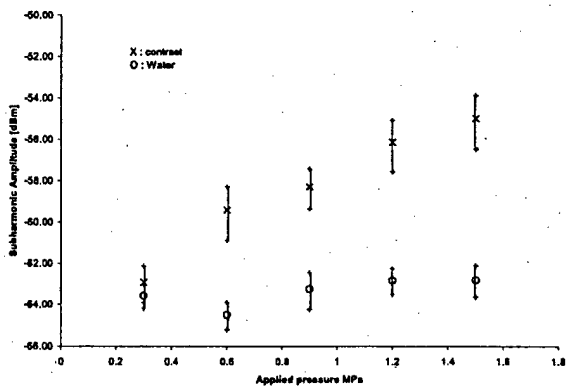

Fig. 4. The variation in the average subharmonic backscatter from the agent and water as a function of the applied pressure. The tube diameter is 1000 μm. The vertical bars indicate $\pm$ 1 SD.

Also, for this experiment, a single straight tube was used. Angiogenesis causes the generation of a large number of tortuous vessels in and around the tumor, ranging from 20 to 300 μm (Li 2000). It has to be noted that it was much more difficult to get the thinner tube exactly in the focal overlap region of the two transducers. Any change in position of the tube would result in a change in the received subharmonic backscatter amplitude. The alignment problems would be significantly less if one were to use a single wide band width transducer for transmitting as well as receiving the US.

Another important factor that may affect the subharmonic amplitude is the velocity of the contrast microbubbles in the tube. The flow velocity in the 300 μm-tube was about 12 cm/s whereas, in the 1 mm-tube, it was 4.9 cm/s. Lower flow velocities generated lesser subharmonic backscatter. This is much higher than the expected flow velocities in the neovessels feeding tumors.

Experiments using the dialyzer show similar subharmonic backscatter. Figure 5 shows a comparison of subharmonic backscatter amplitude from distilled water and contrast microbubbles. An increase in the subharmonic backscatter of about 12 dB was observed when the insonation pressure was increased from 0.3 to 1.5 MPa. The flow velocity in the dialyzer tube was estimated at 2 mm/s, by determining the volumetric flow rate through the dialyzer and dividing by the total cross-sectional area of all the thin tubes. This velocity is close to the actual flow velocity that one might expect to see in neovessels (Li 2000). The actual velocity of flow could not be precisely determined because it would vary depending on the conditions prevailing in the particular tube in the focal region of the two transducers.

One of the major concerns in the dialyzer experiments was the presence of air bubbles trapped between

the tubes of the dialyzer. These relatively large air bubbles would reflect most of the insonation US, causing a reduction in the subharmonic backscatter. Care was taken to remove the air bubbles that could be seen by keeping the cartridge submerged for 24 h prior to commencing of the experiment. Likewise, care was taken that the transducer focal points would overlap and that many tubes of the dialyzer lay within this focal region. Subharmonic backscatter produced from each of these tubes would cause some destructive interference on the receive transducer because the path of propagation would be different.

In both of the experiments, the temporal resolution of the system was low due to the relatively long pulse duration used. Long pulse durations were used to transmit enough power for the generation of subharmonics by volumetric oscillations of the microbubbles. The power spectral density of the received signal showed side bands at the 2-MHz frequency when observing backscatter from the dialyzer. To prevent the transmission of 2-MHz frequency components, a high-pass filter was employed with a cut-off frequency at 2.3 MHz (Fig. 1).

Sufficient subharmonic backscatter was measured from the 300-μm-tube, as well as the dialyzer, indicating that contrast US subharmonic imaging may yield sufficient data for the detection of tumor neovessels. Due to the specificity and resolution that can be achieved by subharmonic US imaging, this could become an important diagnostic tool.

CONCLUSIONS

By using subharmonic imaging with an insonation frequency twice the resonant frequency of the contrast agent, and by using sufficiently high power of insonation, the signal-to-noise ratio of acoustic signals from small vessels (< 300 μm) can be enhanced. The lack of subharmonic backscatter from tissue under normal imaging conditions means that all the subharmonic signals detected are generated from the contrast agent. Hence, the level of subharmonic backscatter is an indication of the level of vascularity of the tissue. The results show that sufficient subharmonic backscatter can be generated by bubbles flowing in vessels of diameter comparable to the ones formed during angiogenesis in tumors. By using suitable instrumentation, this information may be used for the early detection of malignant tumors.

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